Hypersensitive response in potato tuber: elicitation by combination of non-eliciting components from *Phytophthora infestans*

M. J. KURANTZ and R. M. ZACHARIUS

Eastern Regional Research Center,†

Philadelphia, Pennsylvania 19118, U.S.A.

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Insoluble cell wall material and a lipid fraction from mycelia of *Phytophthora infestans* were found to be inactive, separately, as elicitors of the hypersensitive response in potato tuber discs. Recombination of the two materials, however, resulted in a mixture which was able to cause rishitin accumulation as well as necrosis in tuber tissue. The insoluble cell wall material was found to consist of predominately carbohydrate; hydrolysis yielded glucose as the major monosaccharide. Significant levels of protein were also found in the cell wall fraction which indicates that this material was not entirely glucan. The active component(s) of the *P. infestans* lipid material was found in the non-saponifiable fraction and was located by thin-layer chromatography. We suggest that eliciting material from *P. infestans* mycelial extracts requires components other than fungal glucans for activity on potato tuber tissue and describe a system which allows for further investigation of these components.

INTRODUCTION

Potato tuber tissue inoculated with an incompatible race of *Phytophthora infestans* exhibits a resistant or hypersensitive response (HR) [*16*]. Characteristics of the HR include tissue necrosis as well as rishitin accumulation by the host tissue [*17*]. The terpenoids lubimin [*13*], phytuberin [*20*], and spirovetivia 1(10)11-diene-2-one (katahdinone) [*4*] have also been identified in potato tubers inoculated with an incompatible race of *P. infestans*.

An elicitor from cell free extracts of *Phytophthora megasperma* var. *sojae* has been isolated and characterized [*1*]. The cell wall glucan elicitor was found to be non-host specific and was an active elicitor of phytoalexins in non-host plants [*3*]. Cell free sonicates of compatible or incompatible races of *P. infestans* mycelia cause a hypersensitive response in potato tuber tissue including terpenoid accumulation and necrosis [*19*].

An elicitor of the HR in potato tubers from *P. infestans* has not yet been as completely characterized although fungal cell wall glucans have been implicated [*15*]. Highly purified glucans from various sources, including *P. infestans*, however, were reported to be relatively inactive as elicitors [*11*]. In addition, the involvement of fungal lipids has been suggested in the elicitation of the HR by cell free *P. infestans*

†Agricultural Research Science and Education Administration, U.S. Department of Agriculture.

extracts [6]. This paper presents evidence for a role for *P. infestans* lipids as well as cell wall material in the elicitation of the HR by potato tuber tissue.

MATERIALS AND METHODS

Plant material and pathogen

Katahdin potato tubers were used for all assays. Tubers from the 1979 crop were stored continuously at 10 °C and conditioned at room temperature for 24 h before use. *P. infestans* race 0 was grown in bottles of rye steep—2% agar media [*10*] at 20 °C. Cultures, age 10 to 14 days, were flushed with sterile water and the agar surfaces gently rubbed with a glass rod. Fernback flasks (2 800 ml capacity) containing 1500 ml of liquid rye steep media were charged with the suspension of mycelia and sporangia. The flasks were incubated at 21 to 24 °C with shaking at 85 r.p.m. for 14 days.

Fractionation of mycelia

The mycelial mass from the liquid culture was fractionated by a modification of the procedure of Zevenhuizen & Bartnicki-Garcia [*21*].

Mycelia were collected by filtration on a coarse sintered glass funnel, washed with 3 l of water, drained, and weighed. The mycelial mass from 9 l of medium (55·0 g fresh weight) was homogenized in a blender with 300 ml of water for 5 min and sonified for 10 min with a Sonifier Cell Disrupter (Heat Systems-Ultrasonics†) set at full power. The suspension was centrifuged at 1000 ***g*** for 30 min and the supernatant removed. The cell wall fraction was washed 3 times with water by centrifugation and the crude cell wall material was dried by lyophilization. This dried material was labelled as cell wall fraction A (CWF-A). The dried material was subsequently extracted with a total of 1 l chloroform : methanol (2 : 1, v/v). Insolubles were collected by filtration and dried at 110 °C to remove any residual solvents. This material was labelled cell wall fraction B (CWF-B). CWF-B was then stirred in 1% sodium dodecyl sulfate for 1 h at 90 °C. Insolubles were collected by centrifugation and washed 3 times with water by centrifugation. The insolubles were dried by lyophilization and labelled cell wall fraction C (CWF-C). Final purification was accomplished by extracting the cell wall material to remove cellulose as described previously [*21*]. The resultant material corresponds to an insoluble cell wall glucan and is referred to as the *P. infestans* "glucan" throughout this paper.

The chloroform–methanol solubles obtained from the previous procedure were purified by washing with water and salt solution according to the method of Folch *et al.* [7]. The washed *P. infestans* lipid fraction was taken to dryness with a rotary evaporator and the residue was dissolved in 5 ml of chloroform and stored at 4 °C.

Tissue inoculation and rishitin determination

Potato tubers were surface sterilized in 1% NaClO solution for 30 min, rinsed with water, and cut into 0·4 cm thick slices. Discs, 4·0 cm in diameter were removed from

† Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

the center of the slices with a sterile cannular device, rinsed with sterile water and placed in Petri dishes.

P. infestans mycelial fractions were suspended in water and dispersed by use of a vortex mixer. Samples soluble in chloroform were prepared by evaporating the solvent under a stream of N_2 and were suspended in water by use of an ultrasonic bath and vortex mixer. All samples were autoclaved at 120 °C and 15 lbs prior to application to tuber discs. The sterile suspensions were applied at a volume of 0·5 ml per disc. Inoculated discs were incubated at 20 °C for 5 days after which they were graded according to the extent of necrosis visible on the disc surface and finally dried by lyophilization.

Rishitin was determined by grinding the dried discs (10 tuber discs per treatment) with 125 ml of methylene chloride in a blender. The mixture was filtered through a coarse sintered glass funnel and the process repeated. The combined filtrates were concentrated to dryness on a rotary evaporator and the residue dissolved in methanol. The methanolic solution was evaporated to 1 ml, chilled, and centrifuged. The clear supernatants were removed, dried under N_2, and redissolved in methanol (35 µl per g dry wt of tuber tissue).

The methanol extracts were chromatographed on 250 µm silica gel-G thin-layer plates (Analtech Inc.) which were developed in cyclohexane : ethyl acetate (1 : 1, v/v) (Solvent System A). Rishitin yielded a cherry red color at room temperature on spraying the plate with a saturated solution of $SbCl_3$ in chloroform. Quantitative rishitin determinations were carried out according to the g.l.c. method of Heisler *et al.* [*8*].

Chemical methods

Carbohydrate was estimated by the phenol–sulfuric acid method of Herbert *et al.* [*9*]. Where reported, glucose was determined by gas chromatography of the aldononitrile acetates [*18*] following hydrolysis according to the method of Bartnicki-Garcia [*2*]. Protein was estimated by amino acid analysis of the above hydrolysates on a Beckman Amino Acid Analyzer Model 119C. Water soluble samples were assayed for protein by the method of Lowry *et al.* [*12*].

The *P. infestans* lipid fraction was saponified with 2·8 N methanolic KOH under reflux for 20 h (1 part lipid fraction : 10 parts reagent, w/w). Methanol was removed by evaporation and the mixture was diluted with an equal volume of water. The aqueous solution was extracted 3 times with diethyl ether. The ether layer was removed, washed 3 times with water, and dried by addition of anhydrous magnesium sulfate. Ether solubles were obtained by filtration, concentrated to dryness, dissolved in chloroform, and stored at 4 °C.

The *P. infestans* non-saponifiable lipid fraction (NSF) was separated by thin-layer chromatography on silica gel-G plates. Plates were developed in solvent system A and in a mixture of petroleum ether : diethyl ether : acetic acid (90 : 10 : 1) (solvent system B). Visualization of components was effected by spraying the edges of the plates with $SbCl_3$ in chloroform, with 5% phosphomolybdic acid in ethanol, or by exposure to iodine vapors. Zones were removed from the plates by scraping the gel from developed plates. Material was eluted from the silica gel with chloroform : methanol (1 : 1, v/v), extracts were evaporated to dryness and made up to a desired concentration with chloroform.

Enzyme digestion

The *P. infestans* cell wall "glucan" obtained as described earlier, was digested with laminarinase, a β(1→3) glucanase (Calbiochem). Ten mg of the enzyme was dissolved in 25 ml of 0·05 M acetate buffer, pH 5·0, mixed with 200 mg of cell wall "glucan", and incubated at 30 °C for 72 h. Reaction was halted by heating at 100 °C for 10 min. Insolubles were removed by centrifugation and the supernatant was applied to a column of Sephadex G-25 and eluted with water. Fractions giving a positive response for carbohydrate were pooled and concentrated to approximately 10 ml. Material was precipitated by addition of 5 volumes of methanol and the mixture was allowed to stand at 4 °C for 24 h. Precipitate was collected by centrifugation, washed with cold methanol, and dried in a vacuum dessicator.

Recombination of fractions

The lipid fraction, non-saponifiable, and cell wall fractions were recombined and subjected to the disc assay. Recombined samples were prepared by placing the desired volume of chloroform solution in tubes and evaporating the solvent with N_2. Insoluble cell wall materials, in the dry form, were combined with the dried residues, an appropriate volume of water was added, and a suspension obtained by use of an ultrasonic bath and a vortex mixer. Soluble cell wall materials from the enzyme digest were first dissolved in water then recombined with the dried lipid fraction.

In place of the fungal cell wall materials, various recombined samples were prepared using laminarin (Calbiochem), yeast glucan (isolated by the method of Misaki [*14*]), and *P. infestans* lipid fraction. Lipid from rye seeds, from which the *P. infestans* medium was prepared, was extracted with chloroform : methanol (2 : 1, v/v), and combined with the *P. infestans* cell wall "glucan". In all experiments, samples were autoclaved at 120 °C and 15 lb for 30 min before application to tuber discs.

RESULTS

Fractionation of mycelia

The effect of the purification procedure on the ability of cell wall fractions of *P. infestans* mycelia to elicit the HR was monitored by use of the disc assay. The products obtained from a succession of extractions designed to remove all but insoluble cell wall glucans were tested after each step of the procedure. Dried materials were suspended in water and applied to tuber discs at a concentration of 5·0 mg dry weight per disc. Samples were applied to each of 10 discs and reported values are the average of 3 determinations. Fractions which were able to cause both tissue necrosis and rishitin accumulation in tuber discs were considered to be active.

Table 1 shows that crude, water-washed insoluble cell wall material (CWF-A) was active in eliciting the HR in tuber discs. Extraction of this material with chloroform–methanol results in fraction CWF-B which was comparatively less active. Treatment with sodium dodecyl sulfate results in the relatively inactive material CWF-C. The insoluble cell wall "glucan", appeared to be completely inactive when applied to the potato tuber discs.

The table also shows that as the lipid content of the fractions decreased, there was a decrease in rishitin levels and necrosis in inoculated tuber discs.

In vitro recombination of cell wall and lipid fractions

To determine if the removal of the lipid fraction from the cell wall material influences the ability of these materials to elicit the HR, fractions were recombined and tested by application to tuber discs. Fractions CWF-B and CWF-C, which apparently lost activity after the extraction steps, were recombined with the *P. infestans* lipid fraction. The dried wall material was mixed with the fungal lipid fraction at concentrations of 5·0 mg dry weight per tuber disc plus 1·0 mg, 2·0 mg, or 3·0 mg dry weight per tuber discs of lipid. To ascertain if the activity was distributed between the lipid and wall fractions, the lipid fraction alone, at the three concentrations above, was suspended in water and applied to tuber discs.

Table 2 shows that the recombined fractions were active in eliciting rishitin accumulation and necrosis in tuber discs. The lipid fraction alone did not cause necrosis or appreciable rishitin accumulation at the concentrations tested. Eliciting activity appears to be restored to the extracted wall fractions by readdition of the lipid fraction.

The insoluble cell wall "glucan", from *P. infestans* was mixed with the lipid fraction and applied to tuber discs. The concentration of insoluble "glucan" was maintained constant while the concentration of lipid fraction per disc was varied. Figure 1 shows the effect of this combination on the rishitin accumulation in tuber discs. There is an increase in rishitin accumulation as the concentration of the lipid fraction of the "glucan"–lipid mixture is increased. The insoluble "glucan" material at a concentration of 5·0 mg per disc caused no rishitin accumulation or necrosis. The lipid fraction applied alone or a control at the same levels used in the recombination caused no necrosis at any level tested and a trace of rishitin at the two highest

TABLE 1

Effect of purification procedures on the ability of P. infestans *cell wall material to elicit rishitin accumulation and necrosis in potato tuber discs*

Treatment			Rishitin[a]	
Fungal cell wall material	Lipid % of dry wt	mg dry wt per tuber disc	µg per g dry wt tuber tissue	Tissue[e] necrosis
CWF–A	13·0	5·0	178	+++
CWF–B	2·2	5·0	94	++
CWF–C	ND	5·0	TR[c]	—
Insoluble cell wall "glucan"	ND	5·0[b]	ND[d]	—

[a] Values reported are averages of 3 determinations.
[b] 5·0 mg dry wt of insoluble "glucan" is approximately equivalent to 0·25 g fresh wt of mycelia.
[c] TR=Trace, which is ≦1·0 µg rishitin per g dry wt tuber tissue.
[d] ND=None detected.
[e] Necrosis was graded, after 5 days incubation, on a scale from (—) indicating no necrosis to (++++) indicating very extensive necrosis.

levels. The combined lipid–"glucan" fractions also caused an increase in the degree of tissue necrosis as the concentration of the lipid was increased.

The photograph (Plate 1) illustrates the condition of potato tuber slice surfaces when inoculated with the lipid fraction, "glucan"–lipid combination, and "glucan" alone. Tuber slice 1 treated with 3·0 mg dry weight of lipid fraction suspended in water had no surface necrosis and resembled H_2O inoculated controls after 5 days

TABLE 2

Effect of in vitro *recombination of treated* P. infestans *cell wall material and lipid fraction on the elicitation of rishitin accumulation in potato tuber discs*

Treatment					
Fungal cell wall material	mg dry wt per tuber disc		*P. infestans*[b] lipid fraction mg dry wt per tuber disc	Rishitin[c] µg per g dry wt tuber tissue	Tissue[f] necrosis
CWF–B	5·0	+	1·0	141	+++
			2·0	197	+++
			3·0	235	++++
CWF–C	5·0	+	3·0	195·4	+++
Lipid fraction[a] controls			1·0	ND[d]	—
			2·0	ND	—
			3·0	TR[e]	—

[a] Lipid fraction controls indicate application of the lipid fraction to tuber discs with no cell wall material present.
[b] Lipid accounted for 2·43% of the fresh wt of mycelia.
[c] Values reported are averages of 3 determinations.
[d] ND=None detected.
[e] TR=Trace, which is $\leqq 1{\cdot}0$ µg rishitin per g dry wt tuber tissue.
[f] Necrosis was graded, after 5 days incubation, on a scale from (—) indicating no necrosis to (++++) indicating very extensive necrosis.

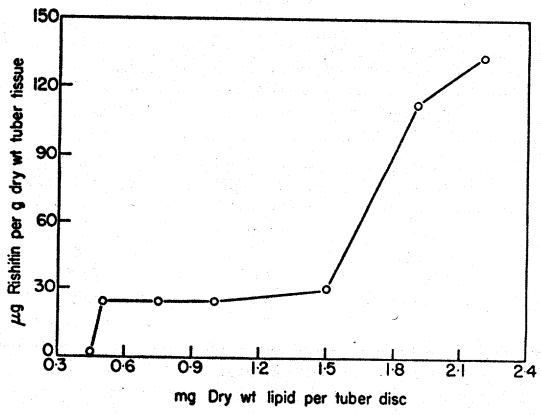

FIG. 1. Effect of *in vitro* recombinations of *P. infestans* insoluble cell wall "glucan" and lipid fraction on the accumulation of rishitin by potato tuber discs. The mixtures contained a constant concentration of *P. infestans* "glucan" equivalent to 5·0 mg dry wt per tuber disc while the concentration of lipid fraction per tuber disc was varied.

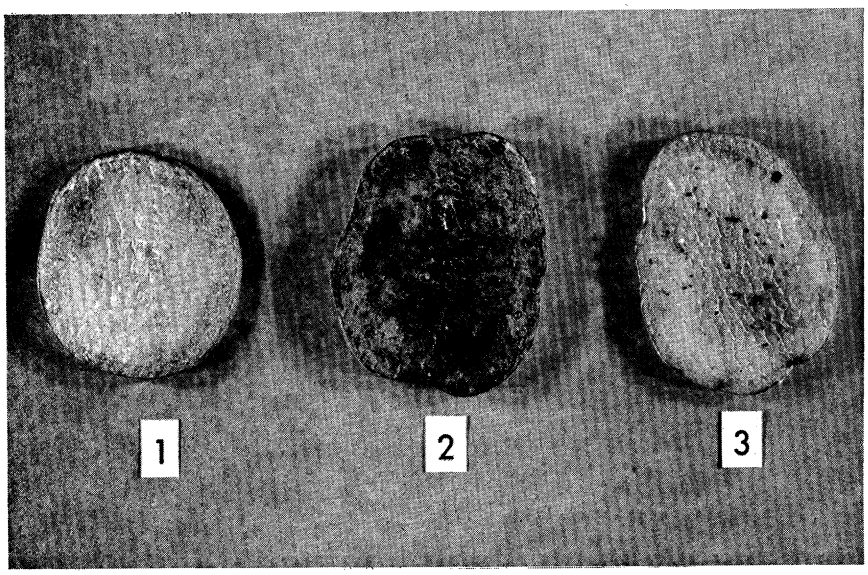

PLATE 1. Potato tuber slices after a 5-day incubation period inoculated with:

(1) *P. infestans* lipid fraction, 3·0 mg dry weight per slice.
(2) *P. infestans* insoluble cell wall "glucan" and lipid fraction mixture, 10·0 mg dry weight "glucan" per slice+3·0 mg dry weight lipid fraction per slice.
(3) *P. infestans* insoluble cell wall "glucan", 10·0 mg dry weight per slice.

incubation. Slice 3 treated with 10·0 mg dry weight of insoluble glucan can be seen to have dark spots over the slice surface. These areas are actually darkened pieces of the insoluble "glucan" material resting on the slice surface. The tissue surrounding these fragments as well as underneath them appeared unaltered after the incubation period. Slice 2 was inoculated with a mixture of insoluble "glucan" and lipid which resulted in a concentration of 10·0 mg dry weight glucan and 3·0 mg dry weight of lipid fraction on the slice. The necrosis was very intense and extended over most of the slice surface particularly around the pieces of insoluble "glucan" material.

The cell wall "glucan" material and lipid fraction were water insoluble but were applied to the tuber discs in water suspensions. In order to discount the possible solubilization of some active component during preparation of the mixture recombinations, a control experiment was performed. This consisted of recombining the cell wall "glucan" with lipid as in Fig. 1, but after autoclaving, the mixtures were centrifuged and the turbid supernatants (from suspended lipid) were applied to tuber discs. No indication of necrotic tissue or rishitin accumulation was found at the end of the incubation period.

Enzyme digestion

A water soluble material obtained by enzyme digestion of the insoluble cell wall "glucan" was tested for the ability to elicit the HR in tuber discs. The soluble fraction was applied to discs alone, and as a mixture with the lipid fraction. The concentrations of individual components was 5·0 mg dry weight per disc of soluble material and 1·0 mg dry weight per disc of lipid fraction. Control discs were the same materials and concentrations applied to tuber discs separately. The soluble fraction caused no rishitin accumulation or necrosis when applied alone to discs. The same was true for the lipid fraction applied alone to tuber discs. The mixture of the two materials, however, resulted in rishitin accumulation of 46·0 μg per g dry weight of tuber tissue and necrosis of (++) on a scale from (—) indicating no necrosis to (++++) indicating extensive necrosis. These results were the average of 3 determinations using 10 discs for each determination.

The composition of the soluble fraction and insoluble cell wall "glucan" is given in Table 3. The water soluble "glucan fragment" which accounted for 19·7% of the dry weight of the insoluble material had an almost identical composition as the insoluble material. Both preparations were predominantly carbohydrate with glucose

TABLE 3

Composition of the P. infestans *insoluble cell wall glucan and soluble fragment prepared by enzyme* digestion

	Total carbohydrate (% of dry wt)	Glucose (% of dry wt)	Protein (% of dry wt)
Insoluble cell wall "glucan"	86·0	78·0	10·5
Soluble "glucan"[a]	88·5	80·0	10·0

[a] Soluble glucan refers to material obtained by enzyme digestion of the insoluble cell wall "glucan".

accounting for the majority of the carbohydrate, however, small quantities of mannose and arabinose were also detected by g.l.c. analysis. The insoluble material as well as the soluble fraction were found to contain some protein. Thus the procedure used to prepare insoluble cell wall glucans from *P. infestans* mycelia did not yield pure glucan.

Recombination of cell wall material with the NSF

The non-saponifiable lipid fraction was recombined *in vitro* with the insoluble cell wall "glucan" and tested for activity on tuber discs. The "glucan" concentration was 5·0 mg dry weight per disc and the NSF concentration per disc was varied. Figure 2 (□) shows the effect of this mixture on the rishitin accumulation in tuber discs when the samples were suspended in water. When NSF–"glucan" was suspended in 0·05% Tween 80 solution the rishitin levels were slightly greater (○). Mixtures of NSF–"glucan" in water suspensions to which the triglyceride, trioctanoin was present at a concentration equivalent to 0·02 mg dry weight per disc, resulted in substantially higher rishitin accumulation by tuber discs (△). The necrosis on disc surfaces was, in general, present but marginal (+) for water and Tween 80 suspensions and fairly extensive (+++) for the mixtures containing the triglyceride.

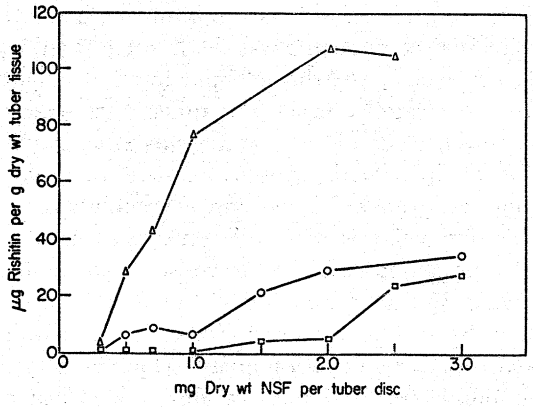

FIG. 2. Effect of *in vitro* recombination of *P. infestans* insoluble cell wall "glucan" and NSF on the accumulation of rishitin by potato tuber discs. The mixtures contained a constant concentration of *P. infestans* "glucan" equivalent to 5·0 mg dry wt per tuber disc while the concentration of NSF per tuber disc was varied. The NSF–"glucan" mixtures were suspended in water (□), in 0·05% Tween 80 (○) and in water with trioctanoin at a concentration equivalent to 0·02 mg dry wt per tuber disc (△).

Requisite controls included 0·5 ml per disc of the 0·05% Tween 80 and 0·02 mg dry weight per disc of trioctanoin. No necrosis or rishitin accumulation resulted from either of these treatments. In addition, mixtures of the above from which the insoluble material was removed by centrifugation also failed to cause a response on tuber discs.

By itself, the NSF at all levels tested on discs failed to elicit necrosis or even trace quantities of rishitin whether suspended in water, Tween 80 or with triglyceride.

Thin-layer chromatography of the NSF

The NSF was applied to silica gel thin-layer plates and developed in solvent system A and B. The active area was found by scraping regions from the plates and testing this material by mixing TLC fractions with "glucan", suspending the material in 0·05% Tween 80 and applying to tuber discs. In solvent system A the active zone was located as a streak between R_F 0·67 and R_F 0·40. In solvent B the active component(s) formed a distinct spot at R_F 0·48. The active area appeared brown after heating when sprayed with $SbCl_3$ and blue after heating, when sprayed with phosphomolybdic acid. The spot appeared rapidly after exposure to iodine vapour at room temperature.

Recombination of known glucans with the lipid fraction

Laminarin, a β(1→3) linked glucan, and yeast glucan, also reported to be a β(1→3) glucan [*14*], were combined *in vitro* with the lipid fraction and applied to tuber discs. The concentrations of each of the two glucans were 5·0 and 10·0 mg dry weight per disc and lipid concentration was 1·0, 2·0, and 3·0 mg dry weight per disc. Controls consisted of the same materials applied alone to tuber discs. The results indicated that the two known β(1→3) glucans were inactive either alone or as mixtures with the lipid fraction in eliciting necrosis or rishitin accumulation.

Rye seed lipid was combined with the insoluble *P. infestans* "glucan" at the same concentration as the above experiment. The rye seed lipid alone on tuber discs caused accumulation of trace quantities of rishitin but no necrosis. When mixed with the *P. infestans* "glucan", again, only trace quantities of rishitin were found and no necrosis observed. None of the above materials appeared to behave as the recombined *P. infestans* fractions.

DISCUSSION

Fractionation of *P. infestans* mycelia by the procedure described in this paper resulted in an insoluble cell wall material and a lipid fraction which separately were not active elicitors of the HR in potato tuber tissues. An active eliciting material was obtained when the lipid fraction and cell wall material were recombined, indicating that both materials are required in eliciting the HR in potato tuber discs.

The insoluble cell wall fraction was predominantly carbohydrate with glucose as the major component but also contained a significant quantity of protein. Because laminarinase was able to digest this fraction, portions of the glucan would appear to consist of β(1→3) linked glucose. Laminarin and yeast glucan, both reported to be largely β(1→3) linked, were not active either alone or in combination with the lipid fraction. This is an indication that the phenomenon observed using the lipid fraction as an elicitor component does not simply depend upon the presence of β(1→3) polysaccharides. This could also suggest that there are other structural differences between these materials and the *P. infestans* material which are required for elicitor activity. In addition, the level of protein found in the insoluble material warrants further investigation as to whether or not this protein is also necessary for elicitor activity in this system.

The water soluble fragment, prepared by enzyme digestion of the insoluble

material, offers a source of material which could be used for further investigations of the structure and function of this portion of the elicitor. This fragment was shown to have a similar composition as the insoluble material and was active on tuber discs only when the lipid fraction was present. In fact, Doke *et al.* [*5*] have recently reported that water soluble glucans from *P. infestans* which do not elicit the HR may be involved in the determination of the compatible interaction between *P. infestans* and potato tuber.

Ersek [*6*] has claimed that lipid from *P. infestans* mycelia was responsible for elicitor activity in cell free mycelial extracts. This was not found to be the case here, although the *P. infestans* lipid fraction and rye seed lipids induced trace quantities of rishitin but no necrosis. Lisker & Kuć [*11*] have reported that some lipids were capable of causing terpenoid accumulation in potato tuber but at levels which were substantially lower than that induced by *P. infestans* elicitor. In addition, the saponified lipid extract, while active in combination with the cell wall material, did not cause production of even trace quantities of rishitin. This may indicate that the trace rishitin levels were caused by the presence of some minor impurity which was subsequently removed by the saponification step.

The data presented here does suggest that some *P. infestans* component other than glucans are necessary for the elicitation of the HR in potato tubers. The active lipid component was heat stable, soluble in chloroform, and active after alkaline hydrolysis. The NSF–"glucan" combination, though active in eliciting rishitin accumulation and necrosis in tuber discs when suspended in water was more effective when the triglyceride, trioctanoin, was present in the mixture. This may have been a result of the trioctanoin, an oily liquid at room temperature, solubilizing the NSF and emulsifying the insoluble residue in the water suspension. Suspensions of the NSF–"glucan" in 0·05% Tween 80 solution were also more active than the water suspended mixture. The triglyceride might, however, be functioning as a solubilizing agent which aids transport of the NSF active constituent(s) through potato tuber cell membranes.

Further work with the *P. infestans* components with which this paper is concerned is necessary for a more complete understanding of the importance of this phenomenon and any possible relationship to the *in vivo* host–pathogen interaction.

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